23394

IN THE U.S. PATENT AND TRADEMARK OFFICE

Inventor	Karoly TIHANYI et al	
Patent App.	10/551,510	
Filed	29 September 2005	Conf. No. 6075
For	PHARMACEUTICAL COMBINATION FOR THE TREATMENT OF SPASTICITY	
Art Unit	1612	Examiner Rogers, J

Hon. Commissioner of Patents
Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 CFR 1.132

We, Pál Kocsis, PhD and István Tarnawa, PhD, both citizens of Hungary, residing respectively at Mezeskalacs ter. 5., H-1155, Budapest, Hungary, and at Kerekgyarton u. 45/C, H-1147, Budapest, Hungary, declare as follows:

THAT we have each awarded respectively by a college or university fully accredited in Hungary the degree of PhD in biological science;

THAT we each have a number of years of experience in the testing of pharmaceutical compositions for anti-spasmodic and analgesic activity in both in vivo and in vitro experimentation;

THAT each of our full curriculum vitae may be attached to this declaration;

THAT we are each Applicants in US Patent Application Serial No. 10/551,510 filed 29 September 2005 and directed to PHARMACEUTICAL COMBINATION FOR THE TREATMENT OF SPASTICITY AND/OR PAIN;

THAT in order to provide evidence in addition to the data presented in the specification in US Patent Application Serial No. 10/551,510, on pages 5 through 12, that the pharmaceutical compositions according to the present invention which contain either tolperisone or eperisone together with dextromethorphan exert a surprising supra additive (synergistic) effect against both spasticity and pain, we have either personally conducted or supervised the carrying out of the following tests:

The key point is the supra-additive synergism between the pharmacological effects of tolperisone and dextromethorphan establiashed in the present application on pages 5 through 12. Now we show some additional pharmacological evidence clearly indicating such an interaction between the two drugs when applied in combination.

Inhibition of spinal reflexes in the rat hemisected spinal cord preparation is a suitable method for evaluating drug effects on spinal neuronal functions having important roles in the effects of e.g. muscle relaxants or analgesic agents. We have performed an <u>in vitro</u> study to compare the spinal action of tolperisone alone and in the presence of dextromethorphan. We measured the reflex inhibitory action of tolperisone by itself and

in the combination. We show the results, and their statistical analysis in the two figures below. Dextromethorphan (0.25 μM) produced little effect. Tolperisone, when applied alone at 25 and 50 μM produced weak and moderate effects, respectively. When the effects of the same concentrations of tolperisone in combination with 0.25 μM dextromethorphan were tested the inhibitions by both combinations were higher than the sums of the effect of dextromethorphan and tolperisone at the corresponding concentrations.

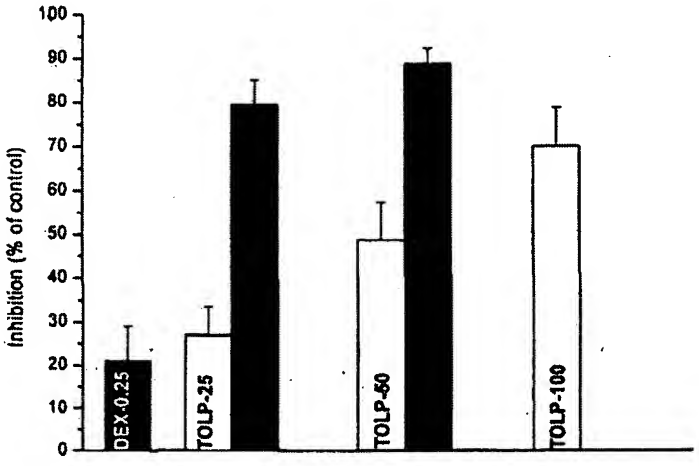

Fig. 1. Effect of tolperisone (TOLP) by itself (white bars) and in the presence of dextromethorphan (DEX; 0.25 μM; gray bars) on the monosynaptic component of the dorsal root stimulation evoked ventral root reflex (MSR). The black bar shows the effect of 0.25 μM DEX by itself. Data represent mean ± S.E.M. values from four experiments. Dextromethorphan significantly increased the effectiveness of tolperisone (ANOVA Duncan's multiple range test, p=0.000189).

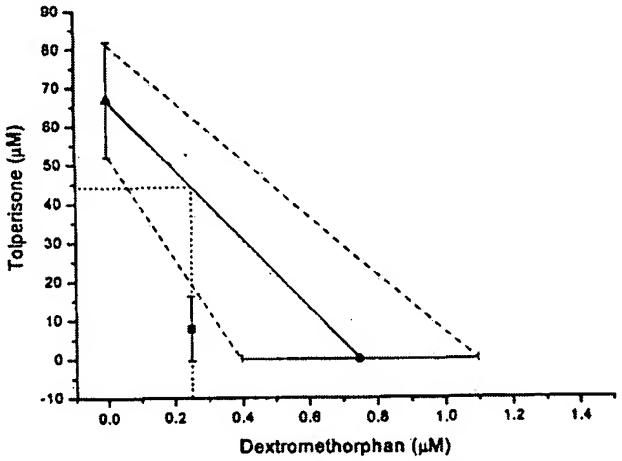

Fig. 2. Isobologram for the interaction between tolperisone and dextromethorphan . The IC_{50} value of tolperisone is plotted on the ordinate (closed triangle) and that of dextromethorphan on the abscissa (closed circle). The IC_{50} values for the inhibition of the MSR were obtained by fitting data to the sigmoid model. The straight line connecting the two plotted IC_{50} values is the isobolographic line, while dashed lines represent 95% confidence intervals. The experimental IC_{50} of the combination of tolperisone and dextromethorphan is plotted as the closed square and 95% confidence intervals as the solid vertical line. If the experimentally determined IC_{50} lies on the isobolographic line, then the drug effects are additive. If the IC_{50} lies below this line, it indicates supra-additivity and when the IC_{50} lies above the isobolographic line, there is infra-additivity. Supposing an additive interaction an IC_{50} of 45 μM is expected for tolperisone when combined with 0.25 μM of dextromethorphan (see dotted lines). However, the actual IC_{50} was almost 6 times less than this (8 μM). There is no overlapping between the 95% confidence intervals, thus the interaction between the two compounds seems to be clearly supra-additive.

The isobologram of the reflex inhibitory action of tolperisone and dextromethorphan, and their combination show a clearly supra-additive effect (synergism) between the two drugs. The statistical comparison (chi square test) of the IC_{50} values of the combinations calculated from the isobologram and those actually determined in the experiments shows a highly significant difference (chi^2=121.1; $p<0.00001$).

THAT based upon the in vitro data presented above and the in vitro and in vivo data that we obtained as presented in Tables 2 through 6 of the present application, we conclude that the combination of either tolperisone or eperisone with dextromethorphan exerts a supra additive (synergistic) anti-spasmodic and analgesic effect;

THAT each of us would also like to point out

In contrast with baclofen (which is a GABAB receptor agonist) or carisoprodol, zoxazolamine, cyclobenzaprine, methocarbamol, or orphenadrine (which are non-specifically acting sedative agents) tolperisone and eperisone cause muscle relaxation via a different, well-characterized mechanism: blockade of neuronal sodium and calcium channels. Thus these compounds, which lack sedative side effect, together with some agents (which are not used in the therapy /silperisone, lanperisone/) represent a sub-family

central muscle relaxants, the "tolperisone-type" (see attached: Kocsis, P.; Farkas, S.; Fodor, L.; Bielik, N.; Than, M.; Kolok, S.; Gere, A.; Csejtei, M.; Tarnawa, I. Tolperisone-type drugs inhibit spinal reflexes via blockade of voltage-gated sodium and calcium channels. J. Pharmacol. Exp. Ther. 315, 1237-1246 (2005)).

The blockade of sodium channels is a prerequisite of the supra-additive synergism between tolperisone and dextromethorphan, which is the subject of our patent application, and no such mechanism-based interaction have been demonstrated between sedative muscle relaxants and dextromethorphan

Muscle relaxant drugs are only useful in alleviating pain associated with muscle spasticity, but are not necessarily effective in the treatment of most pain syndromes, e.g. neuropathic pain. The use of tolperisone and eperisone is restricted to disease areas where pain is caused by abnormally increased muscle tone. Also, The data in BOSE et al, "The efficacy and safety of eperisone in patients with cervical spondylosis: results of a randomized, double-blind, placebo-controlled trial", Methods Find Exp Clin Pharmacol. 1999 April; 21(3): 209 to 213 concerning the analgesic effect of eperisone refer to a situation where the disease of vertebrae (cervical spondylosis) causes painful muscle spasm, which

is responsive to muscle relaxants. The data presented in our patent application prove that tolperisone or eperisone, in combination with dextromethorphan had an anti-allodynic effect in a neuropathic pain model, where muscle spasm do not play a role the development of pain state. This effect suggests a much wider spectrum of analgesic activity.

THAT based upon the difference in the muscle relaxant mechanism of action between tolperisone and eperisone according to the present invention and baclofen, carisoprodol, zoxazolamine, cyclobenzaprine, methocarbamol, or orphenadrine, according to US Patent 5,840,731 to MAYER et al, we further conclude that the supra additive (synergistic) anti-spasmodic activity that we have demonstrated for tolperisone or eperisone with dextromethorphan would not have been predictable or expected from the disclosure in MAYER et al of compositions which contain an analgesic, dextromethorphan, and a muscle relaxant which includes baclofen, carisoprodol, zoxazolamine, cyclobenzaprine, methocarbamol, or orphenadrine;

THAT based upon the disclosure in the prior art, including BOSE et al, that the use of tolperisone and eperisone is restricted to disease areas where pain is caused by abnormally increased muscle tone, our finding that tolperisone or eperisone, in combination with dextromethorphan exerts a supra additive

(synergistic) anti-allodynic effect in a neuropathic pain model, where muscle spasm do not play a role the development of pain state, suggesting a much wider spectrum of analgesic activity, would not at all have been predictable or expected;

THAT we are aware of no information inconsistent with that presented above or which would lead one to a contrary conclusion; and

We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 USC 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

10th November, 2008

Date

Dr. Pál Kocsis

10th November, 2008

Date

Dr. István Tarnawa

Enc: (2) Curriculum vitae

CURRICULUM VITAE

Name: Pál Kocsis PhD

Date of Birth: 05. 14. 1969.
Address at home: H-1155 Budapest, Mézeskalács tér 5.
Phone: 1-416-8247

Address at work: Electrophysiology
Research Division, Gedeon Richter Plc.
19-21 Gyömrői u. H-1103 Budapest, Hungary
Phone: 1-431-5400
Fax: 1-889-8400
Email: p.kocsis@richter.hu

Current position: Research scientist

Education:
From 1995 to 2003 ELTE TTK PhD School of Biology
Neurobiology and Humanbiology
From 1988 to 1994 ELTE TTK biologist

Language skills: German intermediate "C"; ELTE; 1992
English intermediate "C"; Centre for Foreign Languages

Positions:
From 1994 Research scientist at Gedeon Richter Plc

Professional expertise:
Fields: electrophysiology of the spinal cord, voltage gated sodium channels, ionotrop glutamate receptors; centrally acting muscle relaxants, chronic pain, analgesics, hyperactivity disorders; study of drug interactions in various in vitro and in vivo models
Technics: in vitro and in vivo spinal reflex methods, windup methods in rat and rabbit, various chronic pain methods, automated patch clamp (QPatch)

Memberships: Hungarian Neuroscience Society (HNS)
International Brain Research Organization (IBRO)
Federation of European Neuroscience Societies (FENS)
Hungarian Society for Experimental and Clinical Pharmacology
Hungarian Pain Society

Publications:

Number of papers in international journals: 14

1. S. Farkas, Cs. Horváth, P. Kocsis (1995) Some electrophysiological methods for studying functions related to excitatory amino acid receptors. Neurobiology 3(2): 184-185

2. N. Bielik, S. Farkas, P. Kocsis (1997) Studies on the mechanism of action of RGH-5002, a centrally acting muscle relaxant, using whole cell patch clamp technique. Neurobiology 5(1): 43-45

3. S. Farkas, P. Kocsis, N. Bielik (1997) Comparative characterisation of the centrally acting muscle relaxant RGH-5002 and tolperisone and of lidocaine based on their effects on rat spinal cord in vitro. Neurobiology 5(1): 57-58

4. P. Kocsis, S. Farkas, N. Bielik (1997) Participation of NMDA and AMPA type glutamate receptors in spinal segmental reflex: an in vitro study. Neurobiology 5(1): 71-73

5. I. Világi, I. Tarnawa, P. Kocsis, I. Banczerowski-Pelyhe (1998) Effect of glutamate receptor antagonists on excitatory postsynaptic potentials in striatum. Brain Research Bulletin 46(6): 483-486

6. P. Kocsis, I. Tarnawa I, Z Szombathelyi, S Farkas.(2003) Participation of AMPA- and NMDA-type excitatory amino acid receptors in the spinal reflex transmission, in rat. Brain Research Bulletin 60(1-2): 81-91

7. G. Kovacs, P. Kocsis, I. Tarnawa, Cs. Horváth, Z. Szombathelyi, S. Farkas (2004) NR2B containing NMDA receptor dependent windup of single spinal neurons. Neuropharmacology 46:23-30

8. P. Kocsis, G. Kovacs, S. Farkas, Cs. Horváth, Z. Szombathelyi, I. Tarnawa (2004) NR2B receptors are involved in the mediation of segmental reflex potentials but not in the cumulative motoneuronal depolarization in vitro. Brain Research Bulletin 64(2):133-138

9. S. Farkas, P. Berzsenyi, E. Kárpáti, P. Kocsis, I. Tarnawa (2005) Simple pharmacological test battery to assess efficacy and side effect profile of centrally acting muscle relaxant drugs. Journal of Pharmacological and Toxicological Methods 52(2): 264-273

10. P. Kocsis, S. Farkas, L. Fodor, N. Bielik, M. Than, S. Kolok, A. Gere, M. Csejtei, I. Tarnawa (2005)Tolperisone-type drugs inhibit spinal reflexes via blockade of voltage gated sodium and calcium channels. Journal of Pharmacology and Experimental Therapeutics 315(3):1237–1246.

11. I. Tarnawa, H. Bölcskei, P. Kocsis (2007) Blockers of Voltage-Gated Sodium Channels for the Treatment of Central Nervous System Diseases. Recent Patents on CNS Drug Discovery 2: 57-78.

12. M. Than, P. Kocsis, K. Tihanyi, L. Fodor, B. Farkas, G. Kovacs, A. Kis-Varga, Z. Szombathelyi, I. Tarnawa (2007) Concerted action of antiepileptic and antidepressant agents to depress spinal neurotransmission: possible use in the therapy of spasticity and chronic pain . Neurochemistry International. 50(4):642-52.

13. H. Bölcskei, I. Tarnawa, P. Kocsis (2007) Voltage-Gated Sodium Channel Blockers 2001-2006: An Overview. Medicinal Chemistry Research. 15 (1-6), pp. 39-41

14. H. Bölcskei, I. Tarnawa, P. Kocsis (2007) Voltage-Gated Sodium Channel Blockers 2001-2006: An Overview. Medicinal Chemistry Research. 17 (2-7), pp. 356-368

Number of papers in Hungarian: 1

1. P. Kocsis, I. Tarnawa, G. Kovacs, Z. Szombathelyi, S. Farkas (2002) Mydeton: a centrally acting muscle relaxant drug from Gedeon Richter LTD. Acta Pharm Hung 72(1): 49-61

Number of patent applications: 3

1. K. Tihanyi, P. Kocsis, G. Németh, I. Tarnawa, B. Dalmadi (2004) Pharmaceutical combination for the treatment of spasticity and/or pain. WO 2004089352

2. P. Kocsis, I. Tarnawa, M. Thán, K. Tihanyi, G. Németh (2005) Novel pharmaceutical compositions with increased activity. WO 2005058363

3. P. Kocsis, I. Tarnawa, M. Thán, K. Tihanyi, G. Németh (2007) Pharmaceutical compositions comprising a sodium channel blocker in combination with a MAO-B-inhibitor. WO 2007060491

CURRICULUM VITAE

Name: István TARNAWA

Date of Birth: March 06, 1953
Address at home: 1147 Budapest
Kerékgyártó u. 45./c
Hungary
Phone: 36-1-3833140
Email: tarnawa@richter.hu

Address at work:
Research Division, Gedeon Richter Plc.
19-21 Gyömrői út. H-1103 Budapest, Hungary
Phone:
Fax: 36-1-8898400
Email: tarnawa@richter.hu
Current position: Head of Electrophysiology Group

Education:

1996	PhD degree Eötvös Loránd University, Dept. of Physiology,
1987	University doctoral degree (Physiology; Title of dissertation: Electrophysiological Investigations on the Mode of Action of 2,3-Benzodiazepines
1972 - 1977	Eötvös Lorand University, Natural Science Faculty Budapest, Hungary Field of study: Biology (M.S. degree in 1977)

Positions:

1999-present	Head of Electrophysiology Group, Pharmacology and Drug Safety Research, Gedeon Richter Ltd.
1997-1999	Head of the CNS Research Laboratory, Chinoin Pharmaceutical Works Co., Ltd.
1994-1997	Head of the Electrophysiology Laboratory, Department of Neurobiochemistry, Institute for Drug Research (IDR)
1993-1996	Project leader of AMPA antagonist research at IDR
1986-1995	Research Scientist (part-time position), Tissue slice laboratory, Dept. of Comparative Physiology, Eötvös Lorand University, Budapest
1987-1994	Senior staff researcher, 1st Pharmacology Dept, IDR
1982-1987	Research Scientist, 1st Pharmacology Dept, IDR
1977-1982	Research Associate, 1st Pharmacology Dept, IDR

Number of publications: 61 (full list enclosed)
Number of patent applications: 19

Memberships:

Hungarian Physiological Society	1983
Hungarian Neuroscience Society	1985
European Neuroscience Society	1988
International Brain Research Organization	1988
Hungarian Pharmacological Society	1996
Hungarian Pain Society	1999
Advisory Board Member; Recent Patents on CNS Drug Discovery, Bentham Sci.	2006

Professional expertise (experience in research)

1976-1977:	Electrophysiological work in snail giant neurons; Inst. Gerontology, Budapest, supervisor: Dr. S. Tóth.
1977-1981	Pharmacological work with opiates and other newly synthesized analgesic and antiinflammatory compounds; IDR, supervisor: Dr. J.I. Székely.
1982-1983	Electrophysiological investigations on the effect of benzodiazepines in snail neurons; IDR, Supervisor: Dr. F. Andrási.
1983-1985	Effects of newly synthesized neurotropic drugs on single unit activity of the substantia nigra, in anesthetized rats; IDR, Supervisor: Dr. F. Andrási.
1985	Brain slice studies on NMDA receptors, Effects of iontophoretically applied amino acids on lumbar alpha motoneurons in immobilized cats; Inst. Physiol., Univ. Aarhus, Denmark, supervisors: Prof. I. Engberg and Dr. J.A. Flatman.
1986-1987:	Effects of GYKI 52466 on spinal reflexes, in cats, and on evoked potentials in cortical slices; IDR and Dept. Comp. Physiol, Eötvös University, Budapest.
1988	Identification of GYKI 52466 as a selective non-NMDA antagonist, in brain slices; Inst. Physiol., Univ. Aarhus.
1989-1997	Electrophysiological and pharmacological studies on novel derivatives of GYKI 52466 (IDR); Investigation of the role of glutamate receptors in normal physiological neurotransmission (especially in the hippocampus, neocortex, and spinal cord), in synaptic plasticity, and pathological processes (epilepsy, neurodegeneration); IDR, and Eötvös University.
1991	Intracellular electrophysiological study on the role of AMPA receptors in Ia EPSPs of spinal motoneurons, in spinal cats; in Dr. J. Durand's lab, CNRS, Marseille.
1992-1997	Project manager of the 2,3-benzodiazepine AMPA antagonist research program at IDR. Talampanel (GYKI 53773) has been selected as an antiepileptic drug candidate
1994	Whole-cell voltage clamp studies on freshly isolated cerebellar Purkinje cells; in Dr. David Bleakman's laboratory, Lilly Res. Labs. Erl Wood, England.
1995	Acquiring new tissue culture methods, and investigation of glutamate receptors mediated responses in co-cultures of brainstem motoneurons and muscle cells; in the lab of Dr. J.P. Gueritaud, CNRS, Marseille (BALATON program).
1995-1997	Establishment of a new patch clamp laboratory at IDR. Whole-cell clamp studies on the effects of positive and negative AMPA receptor modulators in acutely isolated or cultured neurons, and on recombinant GluR1 receptors.
1997-	Organization of research programs on CNS active compounds. Ion current measurements with whole-cell patch clamping; reflex measurements in the rat hemisected spinal cord model; spinal reflex potential measurements in spinalized rats; wind-up studies in vitro and in vivo; neuronal unit activity measurement; drug induced tremor model. Investigation of drug interaction studies in various in vitro and in vivo models.

ISTVÁN TARNAWA
LIST OF PUBLICATIONS

2008.11.03.

Papers published in peer reviewed journals

J.I. Székely, E. Miglécz, Zs. Dunai-Kovács, I. Tarnawa, A.Z. Rónai, L. Gráf and S. Bajusz (1979) Attenuation of morphine tolerance and dependence by α-melanocyte stimulating hormone (α-MSH). *Life Sci.* 24: 1931-1938.

I. Tarnawa, J.I. Székely (1980) Effect of morphine in combination with chlorpromazine and haloperidol on operant behavior; *Pharmacology* 21: 186-192.

E. Sineger, K. Horváth, E. Miglécz, I. Tarnawa, F. Andrási, J.I. Székely (1982) Morphine and D-met^2, pro^5-enkephalinamide do not show specific neuroleptic activity; *Eur. J. Pharmacol.* 80: 359-368.

S. Farkas, I. Tarnawa, P. Berzsenyi (1989) Effects of some centrally acting muscle relaxants on spinal root potentials; *Neuropharmacology* 28. 161-173.

I. Tarnawa, S. Farkas, P. Berzsenyi, Á. Pataki and F. Andrási (1989) Electrophysiological studies with a 2,3-benzodiazepine muscle relaxant: GYKI 52466; *Eur. J. Pharmacol.* 167. 193-199.

V.V. Senatorov, I. Világi I. Tarnawa, I. Banczerowski-Pelyhe, and Z. Fülöp (1990) Electrophysiological and morphological evidence of functional connection between intracortical neocortex grafts and the host brain (Russian text); *Dokl. Akad. Nauk. SSSR* 315. 1500-1504.

I. Világi, I. Tarnawa, and I. Banczerowski-Pelyhe (1991) Changes in the seizure activity of the neocortex during the early postnatal development of the rat; *Epilepsy Res.* 8. 102-106.

I. Világi, I. Tarnawa, and I. Banczerowski-Pelyhe (1992) Development of LTP in the somatosensory cortex of rats of different ages; *Neurosci. Lett.* 141: 262 - 264.

I. Tarnawa, P. Molnár, L. Gaál and F. Andrási (1992) Inhibition of hippocampal field potentials by GYKI 52466 in vitro and in vivo; *Acta Physiol. Hung.* 79: 163 -169.

I. Tarnawa, P. Berzsenyi, T. Hámori, P. Botka, J. Körösi and F. Andrási (1993) Structure-activity relationships among 2,3-benzodiazepine compounds with AMPA antagonistic action; *Bioorg. Med. Chem. Lett.* 3: 99-104.

I. Engberg, I. Tarnawa, J. Durand, and M. Ouardouz (1993) An analysis of synaptic transmission to motoneurones in the cat spinal cord using a new selective receptor blocker; *Acta Physiol. Scand.* 148: 97-100.

S. Tóth, I. Tarnawa, I. Pénzes, J. Sármány, Zs. Tóth (1994) Age- dependent modification of aspecific cellular effects of the benzodiazepine flunitrazepam; *Arch. Gerontol. Geriatr.* 18: 169-180.

V.V. Senatorov, I. Világi, I. Tarnawa, I. Banczerowski-Pelyhe, Z. Fülöp (1995) Low extracellular magnesium unmasks N-methyl-D-aspartate-mediated graft-host connections in rat neocortex slice; *Neuroscience* 64: 443-458.

E.S. Vizi, Á. Mike, I. Tarnawa (1996) 2,3-benzodiazepines (GYKI 52466 and analogs): Negative allosteric modulators of AMPA receptors; *CNS Drug Rev.* 2: 91-126.

I. Világi,.G. Csúcs, I. Tarnawa, I. Banczerowski-Pelyhe (1996) Involvement of NMDA and non-NMDA receptors in the neocortical synaptic activation: an in vitro study on brain slices of rats of different ages; *Neurosci. Lett.* 203: 139-142.

D. Bleakman, B.A. Ballyk, D.D. Schoepp, A.J. Palmer, C.P. Bath, E.F. Sharpe, M.L. Woolley, H.B. Bufton, R.K. Kamboj, I. Tarnawa, D. Lodge (1996) Activity of 2,3-benzodiazepines at native rat and recombinant human glutamate receptors in vitro: stereoselectivity and selectivity profiles; *Neuropharmacology* 35: 1689-1702.

E.S. Vizi, Á. Mike, I. Tarnawa (1997) The functional study of kainate receptors - hopes and doubts; *TINS* 20: 396.

J.I. Székely, R. Kedves, I. Máté, K. Török, I. Tarnawa (1997) Apparent antinociceptive and antiinflammatory effects of GYKI 52466; *Eur. J. Pharmacol.* 336: 143-154.

I. Világi, P. Kocsis, I. Tarnawa, I. Banczerowski-Pelyhe (1998) Effect of glutamate receptor antagonists on excitatory postsynaptic potentials in striatum; *Brain Res. Bull.* 46: 483-486.

I. Tarnawa and E:S: Vizi (1998) 2,3-Benzodiazepine AMPA antagonists; *Rest. Neurol. Neurosci.* 12: 41-57.

T. Launey, A. Ivanov, G. Kapus, N. Ferrand, I. Tarnawa, J.P. Gueritaud (1999) Excitatory amino acids and synaptic transmission in embryonic rat brainstem motoneurones in organotypic culture; *Eur. J. Neurosci.* 11: 1324-1334.

T. Hámori, S. Sólyom, P. Berzsenyi, F. Andrási and I. Tarnawa (2000) Structural analogues of some highly active non competitive AMPA antagonists; *Bioorg. Med. Chem. Lett.* 10: 899-902.

G. Kapus, I. Bódi, Á. Pataki, J.-P. Gueritaud, J.I. Székely, I. Tarnawa (2000) Differential modulation of the GYKI 53784-induced inhibition of AMPA currents by various AMPA-positive modulators in cerebellar Purkinje cells; *Eur. J. Pharmacol.* 397: 43-47.

G. Ábrahám, S. Sólyom, E. Csuzdi, P. Berzsenyi, I. Ling, I. Tarnawa, T. Hámori, I. Pallagi, K. Horváth, F. Andrási, G. Kapus, L. G. Hársing, Jr., I. Király, M. Patthy and G. Horváth (2000) New non competitive AMPA antagonists; *Bioorg. Med. Chem.* 8: 2127-2143.

G. Kapus, J. I. Székely, J. Durand, A. Ruiz and I. Tarnawa (2000) AMPA receptor antagonists, GYKI 52466 and NBQX, do not block the induction of long-term potentiation in therapeutically relevant concentrations; *Brain Res. Bull.* 52: 511-517.

G. Maksay, L. Fodor, N. Bielik, I. Tarnawa (2001) Bicarbonate and thiocyanate ions affect the gating of γ-aminobutyric acidA receptors in cultured rat cortical cells; *Neurosci. Lett.* 311: 169-172.

S. Sólyom, T. Hámori, A.P. Borosy, I. Tarnawa, P. Berzsenyi (2002) Triazolo- and imidazolophthalazines, New non-competitive AMPA antagonists? *Med. Chem. Res.* 11/1: 39-49.

S. Sólyom, I. Tarnawa (2002) Non-competitive AMPA antagonists of 2,3-benzodiazepine type; *Curr. Pharmaceut. Design* 8: 913-939.

A. Gulyás-Kovács, J. Dóczi, I. Tarnawa, L. Détári, I. Banczerowski-Pelyhe, I. Világi (2002) Comparison of spontaneous and evoked epileptiform activity in three in vitro epilepsy models; *Brain Res.* 945: 174-180.

I. Világi, J. Takács, A. Gulyás-Kovács, I. Banczerowski-Pelyhe, I. Tarnawa (2002) Protective effect of the antiepileptic drug candidate talampanel against AMPA induced neurotoxicity in neonatal rats; *Brain Res. Bull.* 59: 35-40.

P. Kocsis, I. Tarnawa, Z. Szombathelyi, S. Farkas (2003) The participation of AMPA and NMDA type excitatory amino acid receptors in the spinal segmental reflex transmission, in rat; *Brain Res. Bull.* 60: 81-91.

Gy. Kovács, P. Kocsis, I. Tarnawa, Cs. Horváth, Zs. Szombathelyi, S. Farkas (2004) NR2B containing NMDA receptor dependent windup of single spinal neurons; *Neuropharmacology* 46: 23-30.

P. Kocsis, Gy. Kovács, S. Farkas, C. Horváth, Z. Szombathelyi, I. Tarnawa (2004) NR2B receptors are involved in the mediation of segmental reflex potentials, but not in the cumulative motoneuronal depolarization, in vitro; Brain Res. Bull. 64: 133-138.

S. Farkas, P. Berzsenyi, E. Kárpáti, P. Kocsis, I. Tarnawa (2005) Simple pharmacological test battery to assess efficacy and side effect profile of centrally acting muscle relaxant drugs; J. Pharmacol. Toxicol. Meth. 52: 264-273.

P. Kocsis, S. Farkas, L. Fodor, N. Bielik, M. Than, S. Kolok, A. Gere, M. Csejtei, I. Tarnawa (2005) Tolperisone-type drugs inhibit spinal reflexes via blockade of voltage gated sodium and calcium channels; J. Pharmacol. Exp. Ther. 315: 1237-1246.

S, Kolok, J. Nagy, Z. Szombathelyi, I. Tarnawa (2006) Functional characterization of sodium channel blockers by membrane potential measurements in cerebellar neurons: prediction of compound preference for the open/inactivated state; Neurochem. Int. 49: 593-604.

I. Tarnawa, H. Bölcskei, P. Kocsis (2007) Blockers of voltage-gated sodium channels for the treatment of central nervous system diseases; Rec. Pat. CNS Drug Discov. 2: 57-78.

M. Thán, P. Kocsis, K. Tihanyi, B. Farkas, G. Kovács, A. Kis-Varga, Z. Szombathelyi, I. Tarnawa (2007) Concerted action of antiepileptic and antidepressant agents to depress spinal neurotransmission: possible therapeutic utility; Neurochem. Int. 50: 642-652.

H. Bölcskei, I. Tarnawa, P. Kocsis (2008) Voltage gated sodium channel blockers, 2001-2006: An overview; Med. Chem. Res. 17: 356-368.

H. Bölcskei, B. Farkas, P. Kocsis, I. Tarnawa (2008) Recent advancements in anti-migraine drug research: focus on attempts to decrease neuronal hyperexcitability; Rec. Pat. CNS Drug Discov. (IN PRESS)

Papers published as book chapters

J.I. Székely, Zs. Dunai-Kovács, E. Miglécz, I. Tarnawa (1978) The in vivo pharmacology of D-met^2, pro^5-enkephalinamide; In: *Endorphins'78*; Eds.: L. Gráf, M. Palkovits and A.Z. Rónai, Akadémia Kiadó and Excerpta Medica, Budapest, Amsterdam, pp. 319-336.

I. Tarnawa, J.I. Székely, Zs. Dunai-Kovács, E. Miglécz (1979) Behavioural effects of a potent morphine-like pentapeptide: D-met^2, pro^5- enkephalinamide; In: *Opiate receptors and the neurochemical correlates of pain*; Ed.: S. Fürst, Pergamon Press, Akadémia Kiadó, Budapest, pp. 101-108.

J.I. Székely, E. Sineger, K. Horváth, E. Miglécz, I. Tarnawa, F. Andrási (1983) Are morphine and D-met^{2},pro^{5}-enkephalinamide specific neuroleptics?; In: *Neuropeptides and Psychosomatic Processes*; Eds.: Endröczi et al., Akadémia Kiadó, Budapest, pp. 693-699.

J.A. Flatman, I. Tarnawa (1987) Calcium and the action of NMDA on cat and rat neocortical neurones; In: *Excitatory amino acid transmission*; Eds.: T.P. Hicks, D. Lodge and H. McLennan, Alan R. Liss, New York pp. 397-400.

I. Tarnawa, I. Engberg and J.A. Flatman (1990) GYKI 52466 an inhibitor of spinal reflexes is a potent quisqualate antagonist; *In: Amino Acids: Chemistry, Biology and Medicine*; Eds.: G. Lubec and G.A. Rosenthal (Escom Science Publishers B.V., Leiden) pp. 538-546.

S. Farkas, I. Tarnawa, P. Berzsenyi, M. Pátfalusi, F. Andrási, and H. Ono (1992) The role of non-NMDA excitatory amino acid receptors in the mediation of spinal reflexes; *In: Frontiers and New Horizons in Amino Acid Research*; Ed.: K. Takai, Elsevier, pp. 471-175.

J. Sármány, I. Pénzes, S. Tóth, I. Tarnawa, M. Meződy (1993) Age-dependence of nonpecific effects of benzodiazepine (BZ) on identified neurons; *In: Recent Adv. in Aging Sci.*, Eds.: E. Beregi, I.A. Gergely, K. Rajczi (Monduzzi Editore, Bologna, Italy), pp. 155-159.

I. Tarnawa, S. Tóth, J. Sármány, I. Pénzes (1994) Age dependence of the glutamate antagonistic effect of GYKI 52466 in snail neurons; *In: Pharmacology of Aging Process: Methods of Assessment and Potential Interventions*, Eds.: I. Zs.-Nagy, D. Harman, K. Kitani; *Ann. N.Y. Acad. Sci.* 717: 129-136.

I. Tarnawa (2001) Allosterical modulation of AMPA receptors; *In: Excitatory Amino Acids: Ten Years Later*; Eds.: E.A. Cavalheiro, D.D. Schoepp, L. Turski; *(Biomedical and Health Research, Vol. 45);* IOS Press, Amsterdam. pp. 179-190.

I. Tarnawa, B. Farkas, P. Kocsis, L. Dézsi (2008) Possible Drug Targets of Neuronal Hyperexcitability Disorders - Relevance for Stroke in: Recent Advances and New Strategies in Stroke Research, Ed.: Franciska Erdő, Research Signpost, 259-290.

Other papers (not refereed, or published in Hungarian)

P. Berzsenyi, I. Tarnawa, S. Farkas, F. Andrási (1988) Pharmacology of a new centrally acting muscle relaxant; *Pharmacol. Res. Commun.* 20 Suppl.1: 139-140.

I. Tarnawa, S. Farkas, P. Berzsenyi, M. Pátfalusi and F. Andrási (1990) Reflex inhibitory action of a non-NMDA type excitatory amino acid antagonist, GYKI 52466; *Acta Physiol. Hung.* 75 Suppl. 277-278.

I. Banczerowski-Pelyhe, I. Tarnawa, and I. Világi (1991) Mirôl vall az agyszelet: közös pontok a tanulásban és az agykárosító folyamatokban?; *Magyar Tudomány* 1991/3: 249-259.

V.V. Senatorov, I. Világi, I. Tarnawa, I. Banczerowski-Pelyhe, Z. Fülöp (1992) Graft-host glutamatergic neuronal interaction in the neocortex; *Neural Plast. (J. Neural Transpl. Plast.)* 3: 311-312.

Banczerowskiné Pelyhe I., Világi I., Tarnawa I. (1993) Az epilepszia sejtszintü mechanizmusai; *Magyar Tudomány* 7: 810-826.

Tarnawa I. (1994) Neuroprotekció - Nemzetközi szimpózium: A Gyógyszerbiokémiai Szakosztály X. Munkaértekezlete; *Biokémia* 18: 138-140.

I. Tarnawa (1994) GYKI 52466 and its novel analogues: non-competitive AMPA antagonists with 2,3-benzodiazepine structure; *Neurobiology* 2: 185-186.

J. Sármány, I. Pénzes, S. Tóth, I. Tarnawa (1994) Rohypnol nemspecifikus hatásának experimentális vizsgálata óriás neuronokon; *Aneszteziológia és intenzív terápia: A Magyar Aneszteziológiai és Intenzív Terápiás Társaság tudományos folyóirata* 24: 87-96.
P. Somogyi, I. Tarnawa (1995) Molecular biological methods in the research of drugs acting on ionotropic glutamate receptors; *Neurobiology* 3: 201-202.

I. Tarnawa, S. Farkas, P. Berzsenyi, F. Andrási, T. Hámori, I. Ling (1996) 2,3-benzodiazepine AMPA receptor antagonists: Mechanism of action and therapeutical possibilities; *Proc. 2nd Seminar on R&D in the Pharmaceutical Industry of Hungary and Korea*, pp. 37-40.

P. Berzsenyi, I. Tarnawa, F. Andrási, S. Farkas (1996) In vivo pharmacological actions of 2,3-benzodiazepine AMPA antagonists; *Proc. 2nd Seminar on R&D in the Pharmaceutical Industry of Hungary and Korea*, pp. 81-86.

P. Kocsis, I. Tarnawa, Gy. Kovács, Zs. Szombathelyi, S. Farkas (2002) Mydeton: a Richter centrális támadáspontú izomrelaxánsa; Acta Pharmaceut. Hung. 72: 49-61. (Hungarian)

0022-3565/05/3153-1237–1246$20.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Copyright © 2005 by The American Society for Pharmacology and Experimental Therapeutics
JPET 315:1237–1246, 2005

Vol. 315, No. 3
89805/3062248
Printed in U.S.A.

Tolperisone-Type Drugs Inhibit Spinal Reflexes via Blockade of Voltage-Gated Sodium and Calcium Channels

Pál Kocsis, Sándor Farkas, László Fodor, Norbert Bielik, Márta Thán, Sándor Kolok, Anikó Gere, Mónika Csejtei, and István Tarnawa

Gedeon Richter Ltd., Pharmacology and Drug Safety Research, Budapest, Hungary

Received May 26, 2005; accepted August 25, 2005

ABSTRACT

The spinal reflex depressant mechanism of tolperisone and some of its structural analogs with central muscle relaxant action was investigated. Tolperisone (50–400 μM), eperisone, lanperisone, inaperisone, and silperisone (25–200 μM) dose dependently depressed the ventral root potential of isolated hemisected spinal cord of 6-day-old rats. The local anesthetic lidocaine (100–800 μM) produced qualitatively similar depression of spinal functions in the hemicord preparation, whereas its blocking effect on afferent nerve conduction was clearly stronger. In vivo, tolperisone and silperisone as well as lidocaine (10 mg/kg intravenously) depressed ventral root reflexes and excitability of motoneurons. However, in contrast with lidocaine, the muscle relaxant drugs seemed to have a more pronounced action on the synaptic responses than on the excitability of motoneurons. Whole-cell measurements in dorsal root ganglion cells revealed that tolperisone and silperisone depressed voltage-gated sodium channel conductance at concentrations that inhibited spinal reflexes. Results obtained with tolperisone and its analogs in the [^{3}H]batrachotoxinin A 20-α-benzoate binding in cortical neurons and in a fluorimetric membrane potential assay in cerebellar neurons further supported the view that blockade of sodium channels may be a major component of the action of tolperisone-type centrally acting muscle relaxant drugs. Furthermore, tolperisone, eperisone, and especially silperisone had a marked effect on voltage-gated calcium channels, whereas calcium currents were hardly influenced by lidocaine. These data suggest that tolperisone-type muscle relaxants exert their spinal reflex inhibitory action predominantly via a presynaptic inhibition of the transmitter release from the primary afferent endings via a combined action on voltage-gated sodium and calcium channels.

2-Methyl-1-(4-methylphenyl)-3-(1-piperidinyl)-1-propanone hydrochloride (tolperisone) is an old, centrally acting muscle relaxant drug that is mainly used for treating muscle spasticities of neurological origin and painful muscle spasms due to rheumatologic conditions. Besides being an effective antispastic agent (Pratzel et al., 1996; Dulin et al., 1998), tolperisone also has analgesic activity in rodents (Sakaue et al., 2004) and in humans (Svensson et al., 2003). It possesses relatively few side effects in humans (Dulin et al., 1998). Other propiophenone muscle relaxants include 1-(4-ethylphenyl)-2-methyl-3-(1-piperidinyl)-1-propanone hydrochloride (eperisone), which is also a registered drug (Bose, 1999), and (−)-2-(*R*)-methyl-3-(1-pyrrolidinyl)-1-[4-(trifluoromethyl)phenyl]-propanone hydrochloride (lanperisone; Sakitama et al., 1997) and 1-(4-ethylphenyl)-2-methyl-3-(1-pyrrolidinyl)-1-propanone hydrochloride (inaperisone; Morikawa et al., 1992), two agents that had been tested in human phase III studies but not introduced into the clinical practice. 1-[(4-Fluorobenzyl)dimethylsilylmethyl]piperidine hydrochloride (silperisone), a sila analog of tolperisone (Farkas et al., 2005), has been shown in mice to have better separation between the desirable effects (i.e., reduction of abnormally increased muscle activity in various models) and anticipated undesirable effects (i.e., central nervous system depression and impairment of voluntary motor control) than currently available centrally acting muscle relaxant drugs. The development of this compound has been discontinued because of an unacceptable side effect found in the chronic toxicity studies.

Only few reports dealing with the mechanism of action of tolperisone-like compounds have been published. Ono et al. (1984) have shown that tolperisone and eperisone exert a

Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
doi:10.1124/jpet.105.089805.

ABBREVIATIONS: TTX, tetrodotoxin; DRG, dorsal root ganglion; NMDA, *N*-methyl-D-aspartate; [^{3}H]BTX, [^{3}H]batrachotoxinin A 20-α-benzoate; ACSF, artificial cerebrospinal fluid; DR-VRP, dorsal root stimulation-evoked ventral root potential; AFP, afferent fiber potential; MN, motoneuron stimulation-related compound action potential; MS, monosynaptically evoked action potential of motoneurons; PAF, primary afferent fiber; ES, extracellular solution; IS, intracellular solution; MSR, monosynaptic reflex; EPSP, excitatory postsynaptic potential; DSR, disynaptic reflex; PSR, polysynaptic reflex; GYKI 52466, 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine hydrochloride.

local anesthetic-like (membrane-stabilizing) action both on motoneurons and primary afferents in vivo as well as on peripheral nerves of rats in vitro. Tolperisone was found to inhibit action potential propagation on both A- and C-fibers of rat sciatic nerve (Quasthoff et al., 2003). The local anesthetic action of tolperisone and eperisone was implicated in their antinociceptive effects on acute pain in mice (Sakaue et al., 2004). Thus, the effect of tolperisone seems to be similar to that of lidocaine (lignocaine; ω-diethylamino-2,6-dimethylacetanilide), which is known to inhibit voltage-dependent sodium currents. Indeed, molecular modeling studies revealed that tolperisone can bind to the same site to which lidocaine can bind (Fels, 1996). In a two-electrode voltage-clamp study in *Xenopus* oocytes, Quasthoff et al. (2003) found an inhibition of both $Na_v1.6$ [tetrodotoxin (TTX)-sensitive] and $Na_v1.8$ (TTX-resistant) recombinant sodium channels by tolperisone. Similar to lidocaine, both silperisone (Düring and Koppenhöfer, 2001) and tolperisone (Hinck and Koppenhöfer, 2001) inhibited sodium currents of frog-isolated Ranvier nodes. The results of the latter study, however, suggest a significant involvement of potassium channels as well in the mediation of tolperisone's action; therefore, the authors concluded that tolperisone cannot be regarded as having a lidocaine-like action, because the latter drug has negligible effect on potassium channels. Furthermore, voltage-clamp studies in snail neurons (Novales-Li et al., 1989) demonstrated an inhibition of voltage-dependent calcium currents by tolperisone and some of its analogs. The close chemical similarity of eperisone, lanperisone, and inaperisone (and to some extent silperisone) to tolperisone suggests a similar mode of reflex inhibitory action.

For better understanding of the mechanism of suppressant action of tolperisone and its analogs on spinal reflexes, the effects of these agents on the spinal reflex machinery between the stimulated dorsal root and the ventral root conveying efferent discharges were studied in the rat spinal cord both in vitro and in vivo, in comparison with the local anesthetic lidocaine. The sodium channel blocking effects of these compounds were characterized in electrophysiological experiments on dorsal root ganglion (DRG) cells, in a functional assay using fluorescent membrane potential dyes, in cerebellar cultures, and in a radioligand binding assay using brain cortical synaptosomal preparation. Their effect on voltage-gated calcium channels was also analyzed.

Materials and Methods

Materials. Silperisone HCl, eperisone HCl, tolperisone HCl, inaperisone HCl, lanperisone HCl, and pipecuronium bromide were synthesized at Gedeon Richter Ltd. (Budapest, Hungary). Lidocaine was obtained from EGIS Pharmaceuticals (Budapest, Hungary). *N*-Methyl-D-aspartate (NMDA) was purchased from Sigma-Aldrich (St. Louis, MO), and TTX was from Latoxan (Valence, France). [^{3}H]Batrachotoxinin A 20-α-benzoate ([^{3}H]BTX; specific activity, 50 Ci/mmol) was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). Scorpion venom (*Leiurus quinquestriatus* from North Africa), bovine serum albumin, choline chloride, sucrose, and HEPES were purchased from Sigma-Aldrich (Budapest, Hungary). Aconitine was purchased from Fluka (Buchs, Switzerland). Salts and other chemicals for artificial cerebrospinal fluid (ACSF) and electrode-filling solutions were purchased from Sigma-Aldrich. Culture medium components were obtained from Invitrogen (Carlsbad, CA). The fluorimetric imaging plate reader membrane potential assay kit (blue) was purchased from Molecular Devices (Sunnyvale, CA).

For intravenous infusion, Rindex solution (68 mM NaCl, 3.5 mM KCl, 1.25 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 555 mM glucose) was purchased from Human Ltd. (Budapest, Hungary). The anesthetic solution for in vivo studies contained 0.25% α-chloralose and 10% urethane (both from Sigma-Aldrich) dissolved in distilled water. The cannula for blood pressure monitoring was filled with saline containing 200 IU/ml heparin (Gedeon Richter Ltd.).

Animals. Wistar rats (bred at Gedeon Richter Ltd. or purchased from Toxicoop, Budapest, Hungary or Harlan, Indianapolis, IN) were used in all the studies. For the in vitro experiments, spinal cords and DRG cells were isolated from 6-day-old male rat pups. Male rats weighing 280 to 320 g were used for the in vivo experiments. The animal room was thermostated at 21 ± 1°C and illuminated artificially from 6:00 AM to 6:00 PM. The rats had free access to food and water. All of the procedures conformed to the guidelines of the National Institutes of Health for the care and use of laboratory animals and were approved by the Institutional Ethical Committee.

Isolated Hemisected Spinal Cord Preparation in Vitro. Rat pups weighing 13 to 16 g were anesthetized with ether and then placed on crushed ice to cool down the spinal cord until the respiration of the animal stopped. The spinal cord was removed and hemisected along the midline. Hemicords were transferred into a storage chamber, and they were incubated at room temperature (23–26°C) in standard ACSF (124 mM NaCl, 3.5 mM KCl, 1.23 mM NaH_2PO_4, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 26 mM $NaHCO_3$, and 10 mM glucose) bubbled with carbogen (95% O_2 and 5% CO_2) for at least 30 min. One hemicord was placed into the recording chamber and perfused at 10 ml/min with ACSF at 26°C (regulated) if not stated otherwise. Glass suction electrodes were used both for stimulation and recording. Dorsal root stimulation-evoked ventral root potentials (DR-VRPs) were recorded from the L5 ventral root. The L5 dorsal root was stimulated with square-wave anodic current pulses (0.2 mA; 0.1 ms; supramaximal for all recorded potential components) at a frequency of 2 min^{-1}. However, before and at appropriate times after drug applications, the input-output relationship (curve) was also determined using gradually increasing current intensities (0.01–0.2 mA). Evoked compound action potentials (afferent fiber potentials; AFPs) were recorded from the spinal cord surface with a glass suction electrode attached to the adjacent L4 dorsal root. In these experiments, a distance of at least 5 mm was kept between the site of stimulation and the dorsal root entry zone.

The first 200 ms of DR-VRP was analyzed. Responses were band-pass-filtered (0.02 Hz–10 kHz), amplified, and fed into a PC via an A-D converter (Digidata 1200; Molecular Devices, Sunnyvale, CA; sampling rate, 10 kHz). A custom-made computer program (Stimulat) controlled both stimulation and data acquisition and performed the on-line data analysis. Evoked potentials were displayed and stored for later evaluation. Different components of the ventral root reflex response were separated according to their poststimulus latencies and durations. The drugs were added into the ACSF only when the measured parameters had become stable. Drug effects were determined when a steady-state inhibition had developed, which was dependent on the drug used (typically after 60–90 min of perfusion).

Standard Surgery for the in Vivo Studies in Spinal Animals. The method was essentially similar to that described previously in more detail (Farkas and Ono, 1995). Rats anesthetized with a mixture of chloralose (25 mg/kg i.p.) and urethane (1 g/kg i.p.) were used. The vagal nerves were severed, and the common carotid arteries were ligated bilaterally at the cervical region. Blood pressure was monitored via a cannula in the carotid artery. The femoral vein was also cannulated to allow intravenous injections. A tracheal cannula was inserted, and the animals were artificially ventilated throughout the experiment. The spinal cord was infiltrated with lidocaine (1%; 50 μl) and transected at the C1 level. The animals were fixed in a spinal stereotaxic frame, and a dorsal laminectomy was performed on vertebrae L1–L6. A pool was formed from the skin of the back and filled with warm paraffin oil. Rectal and oil pool temperatures were

maintained at 36 ± 0.2°C using two heating lamps. During the experiment, Rindex solution was infused (10–20 ml · kg^{-1} · h^{-1}) to maintain the mean arterial blood pressure of spinal animals at around 60 mm Hg.

In Vivo Spinal Reflex Study. Ventral and dorsal roots below L4 were cut bilaterally, L5 dorsal and ventral roots on both sides were isolated, and an ipsilateral pair of them was placed on bipolar silver wire hook electrodes. The dorsal root was stimulated by single impulses (stimulus strength, supramaximal voltage; pulse width, 0.05 ms; and frequency, 10/min). The first 10 ms of the ventral root reflex recorded using a differential amplifier was displayed, stored, and evaluated using the Stimulat software.

Study of Afferent Nerve Conduction in Vivo. In addition to the standard surgery, the right sciatic nerve was exposed in the femoral-popliteal region and placed on a bipolar silver wire electrode. A pool was formed from the skin of the back and of the leg and filled with warm paraffin oil. The sciatic nerve was stimulated by single square-wave impulses (stimulus strength, supramaximal for all A-fibers; i.e., 5–30 V; pulse width, 0.1 ms; and frequency, 10/min). The proximal end of L5 dorsal root transected at the dorsal root entry zone was placed on a silver wire bipolar hook electrode and crushed between the two hooks for monophasic recording of the arriving compound action potential of the afferent nerve fibers.

Motoneuron Excitability Test in Vivo. Excitability of the motoneuron soma and that of the primary afferent fibers was measured similarly to the technique described by Ono et al. (1979). Anesthetized animals were paralyzed with pipecuronium bromide (100 μg/kg starting dose + 50 μg · kg^{-1} h^{-1} in infusion). A tungsten microelectrode, insulated with the exception of its tip, was inserted into the L5 motoneuron pool, which was stimulated by negative pulses (stimulus strength, 0.2–0.5 mA; pulse width, 0.05 ms; and frequency, 10/min). The compound action potential evoked by direct stimulation of motoneurons (MN; first peak) and the potential caused by (mono)synaptic activation of motoneurons (MS; second peak) were recorded from the L5 ventral root. The antidromic action potential, which reflects excitability of the primary afferent fibers (PAFs), was recorded from the L5 dorsal root. Tip position and stimulus strength were finely adjusted to yield similar amplitudes of MN and MS. Data acquisition and analysis in the in vivo studies were performed similarly to those described in the in vitro experiments, except that a higher (25 kHz) sampling rate was used.

Whole-Cell Current Measurements. DRG cells were acutely dissociated from rat DRG of 6-day-old male rats (Roy and Narahashi, 1992). Cells were plated on sterilized glass coverslips previously coated with poly-D-lysine. Cultures were kept in 2% serum-supplemented Dulbecco's modified Eagle's medium at 37°C in 5% CO_2. Inward currents were recorded by the whole-cell patch-clamp technique 1 day after the plating of the cells. Coverslips with the attached neurons were transferred to a recording chamber and constantly superfused with the extracellular solution (ES) at room temperature (22–25°C). The ES used in the sodium current experiments contained 70 mM NaCl, 70 mM choline chloride, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM HEPES, 5 mM HEPES-Na, 0.01 mM $CdCl_2$, and 20 mM glucose, pH 7.35. The ES for calcium current measurements contained 143 mM choline chloride, 5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 20 mM glucose. Patch electrodes (resistances, 1.5–2.5 MΩ) pulled from borosilicate capillary glass were filled with intracellular solution (IS). The composition of IS in the sodium current experiments was 130 mM CsF, 15 mM NaCl, 10 mM tetraethylammonium chloride, 0.1 mM $CaCl_2$, 2 mM $MgCl_2$, 2 mM ATP-Na_2, 10 mM HEPES, and 1 mM EGTA, pH 7.25. The IS for calcium current measurements contained 110 mM CsCl, 4.5 mM $MgCl_2$, 9 mM HEPES, 9 mM EGTA, 4 mM ATP-Na_2, 0.3 mM GTP, 14 mM creatine phosphate, and 50 U/ml creatine phosphokinase, unless indicated otherwise. Osmolarities of ES and IS were 310 and 290 mOsm, respectively. Sodium currents were evoked by 8-ms-long rectangular step depolarizations to 0 mV from different holding potentials at 10-s intervals. Calcium currents were elicited by 20-ms step pulses to 0 mV from a holding potential of −80 mV. An Axopatch 200A amplifier and the pClamp 8.0 software (Molecular Devices, Sunnyvale, CA) were used for recording and analysis. Capacitive transients were compensated; series resistance compensation was also always performed. Test compounds dissolved in the ES were applied onto the cells via multibarreled ejection pipettes controlled by electromagnetic valves. Currents were recorded from fast-kinetics tetrodotoxin-sensitive DRG cells in which application of TTX (0.3 μM) caused an at least 85% inhibition of the sodium current peak amplitude. The availability curves of the sodium current were fitted with the Boltzmann function: $I_{Na} = I_{Na,max}/(1 + \exp([V_H - V_{H1/2}]/k_H))$, where I_{Na} is the peak current amplitude, $I_{Na,max}$ is the maximum available sodium current (the upper asymptote of the fitted sigmoid curve), V_H is the applied holding potential, $V_{H1/2}$ is the holding potential at half-maximal availability, and k_H is the slope factor or width.

Fluorescent Membrane Potential Measurements. Average membrane potential in rat primary cerebellar cell cultures was monitored by fluorimetry. In brief, primary cerebellar cell cultures were initiated from 4-day-old rats. After decapitation, the cerebellum was removed and incubated with 0.25% trypsin for 3 min. After a brief centrifugation (125*g*; 5 min), the cells were resuspended in culture medium (Dulbecco's modified Eagle's medium) containing 10% fetal bovine serum, 20 ng/ml nerve growth factor, 20 mM KCl, 2.5 μg/ml amphotericin B, 100 U/ml penicillin G, and 100 μg/ml streptomycin, and they were plated onto poly-D-lysine-coated 96-well plates (1–2 × 10^5 cells/well). Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air and used for the measurement after 7 days in vitro. Membrane potential was monitored using the fluorimetric imaging plate reader membrane potential assay kit (Molecular Devices) and FlexStation II (Molecular Devices), a plate reader fluorimeter with integrated eight-channel fluid addition capability. Cells were loaded with the fluorescent dye (100 μl/well) by incubating the plate for 20 to 60 min at 37°C. After loading, 50 μl of ACSF (control) or 50 μl of 4× concentrated test compound solution (dissolved in ACSF) was added to each well, and the plate was incubated at 37°C for an additional 10 min. Fluorescence measurements were carried out at 37°C. The dye was excited at 530 nm, and emission was sampled at 565 nm at 1.4-s intervals. After recording baseline for 15 s, 50 μl of 4× concentrated veratridine solution was added to the cells using the pipetter of FlexStation, and fluorescence was monitored for an additional 105 s. This 2-min protocol was executed column by column on the whole plate. The concentration of veratridine (defined as EC_{80}) was determined on each experimental day by performing dose-response measurements with veratridine using cells from the same plating. Raw fluorescence data were expressed as $\Delta F/F$ values (fluorescence change normalized to baseline). The effects of blockers at various concentrations were quantified as percentage of inhibition of the control veratridine response.

[^{3}H]BTX Binding. Rat cerebrocortical synaptosomes were prepared as described by Catterall et al. (1981) with minor modifications. In brief, the cortices of 7- to 8-week-old male rats were dissected and homogenized in 10 volumes of ice-cold sucrose buffer containing 5 mM K_2HPO_4, pH 7.4. The homogenate was centrifuged at 1000*g* (15 min; 4°C), and the resultant supernatant was retained and centrifuged at 20,000*g* (15 min; 4°C). Aliquots of the synaptosomal preparation were frozen on dry ice and stored at −80°C until use. Before use, aliquots were thawed at room temperature and centrifuged in a 10-fold volume of the same sucrose buffer (20,000*g*; 15 min; 4°C). The final pellet containing synaptosomes was resuspended in 4 volumes of Na^+-free medium containing 130 mM choline chloride, 5.5 mM sucrose, 0.8 mM $MgSO_4$, 5.4 mM KCl, and 50 mM HEPES, pH 7.4.

Aliquots (100 μl) equal to approximately 6 to 8 mg/ml protein were used in [^{3}H]BTX binding experiments. Binding assays were performed in the presence of 5.0 nM [^{3}H]BTX, 1 μM tetrodotoxin, 4.0 μg of scorpion toxin, and various concentrations of the added drugs at 37°C for 60-min incubation time. Nonspecific binding was deter-

mined in the presence of 300 μM aconitine. The reaction was terminated by rapid filtration using a UniFilter-96 GF/B (PerkinElmer Life and Analytical Sciences). The filtration plates were washed five times with ice-cold wash buffer containing 5 mM HEPES, 130 mM choline chloride, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, and 0.01% bovine serum albumin. Radioactivity trapped on a 96-well filtration plate was measured by liquid scintillation spectrometry in 40 μl of Microscint 20 scintillation cocktail (PerkinElmer Life and Analytical Sciences) using a TopCount NXT microplate scintillation and luminescence counter (PerkinElmer Life and Analytical Sciences).

Statistics. Data are presented as mean ± S.E.M. For IC_{50} determinations, sigmoidal fitting to parametric data were applied using Origin 6.0 (OriginLab Corp., Northampton, MA).

Results

Isolated Hemisected Spinal Cord in Vitro. A typical DR-VRP (Fig. 1A) consisted of a biphasic population spike (monosynaptic reflex, MSR; time to peak, 7.0 ± 0.4 ms) superimposed on the early phase of a tonic, long-lasting potential shift, reaching a maximum at 10 to 15 ms following the stimulation of the dorsal root. This latter potential basically represents population excitatory postsynaptic potential (EPSP) of motoneurons (Siarey et al., 1992). Nevertheless, small waves attributable to asynchronous firing of motoneurons were often discernibly superimposed on this tonic potential. When the stimulus intensity dependence of the evoked response was investigated (Fig. 1B, control curves), current intensities causing half-maximal activation of MSR, EPSP, and late EPSP were 0.05, 0.045, and 0.03 mA, respectively, whereas 0.2 mA was enough to maximally activate all components. This latter intensity, which was used in the experiments where drugs were tested, caused only negligible activation of C-fibers (not illustrated). Thus, C-fibers do not seem to participate in the generation of the first 180 ms of DR-VRP, including its highly NMDA antagonist-sensitive "tail part" (80–180 ms; Kocsis et al., 2003). This conclusion is in agreement with that of Thompson et al. (1992), who also found a predominant role of A-type afferent fibers in DR-VRP. Increasing the stimulus intensity above supramaximal for Aδ did not result in further increase in any of these response components.

Tolperisone (50–400 μM), eperisone, lanperisone, inaperisone, and silperisone (25–200 μM) as well as lidocaine (200–800 μM) caused concentration-dependent depression of all studied components of DR-VRP (Fig. 2). As illustrated by the action of silperisone in a representative experiment (Fig. 1A), MSR and the tail of EPSP were the most sensitive parameters, whereas the early part of EPSP was apparently less attenuated. Apart from responses just above the threshold, the depression afforded by different doses of silperisone was not dependent on the stimulus strength. Namely, maximum responses of the input-output curves were attenuated, rather than a rightward shift of the curve (Fig. 1B). Hence, these depressant drug effects were unlikely to be the consequence of an elevated excitation threshold of primary afferent axons at the site of stimulation.

Tolperisone, silperisone, and lidocaine all dose dependently inhibited AFP in the concentration range that also depressed EPSP. To be able to make a precise comparison between drug effects on AFP and synaptic responses, we recorded the two activities in the same hemisected spinal cord preparation. To eliminate the interference caused by spiking of motoneurons (MSR), we recorded the EPSP-related component at an elevated temperature, at which excitability of motoneurons in the hemisected spinal cord preparation was depressed (Brooks et al., 1955). Elevation of the temperature of the bathing solution from 26 to 32 ± 1°C depressed MSR, allowing us to record the main component of the incoming AFP (Fig. 3A) and a nearly pure EPSP-related potential (Fig. 3B) simultaneously. Figure 3 shows that lidocaine was clearly more effective in blocking nerve conduction than EPSP, whereas silperisone had an opposite preference regarding the two potentials. Tolperisone was in between the two other drugs, having similar efficacies to block EPSP and AFP.

In Vivo Spinal Reflex Study. A typical ventral root reflex response to stimulation of the dorsal root and its attenuation by silperisone are shown in Fig. 4A. We differentiated three characteristic components of the ventral root reflex potential (as described previously in Farkas and Ono, 1995): monosynaptic, disynaptic, and polysynaptic reflex (MSR, DSR, and PSR, respectively; Fig. 4A). Silperisone (10 mg/kg i.v.) attenuated all of the three components with a

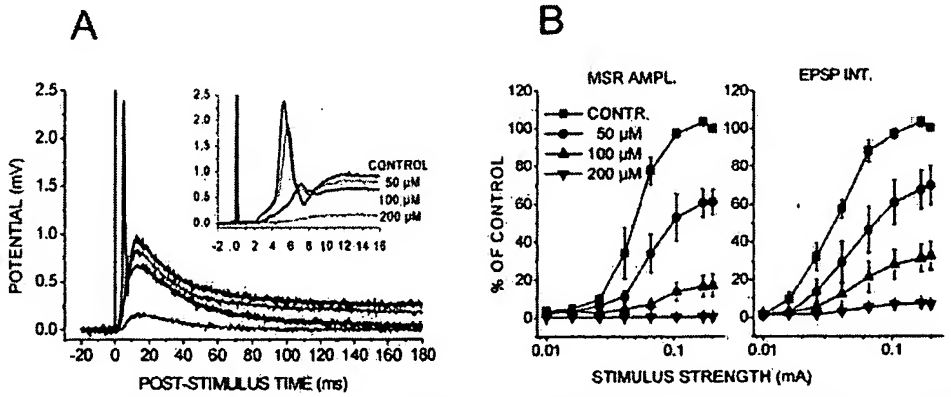

Fig. 1. Effects of silperisone on DR-VRP components in vitro. A, inhibition of DR-VRP by increasing doses of silperisone (50–200 μM) added to the perfusing medium. The inset shows the same potentials on an extended time scale to allow the observation of the changes in the monosynaptic reflex. B, effect of 50 (●), 100 (▲), and 200 μM (▼) silperisone compared with control (■) on the stimulus strength dependence of the peak-to-peak amplitude of monosynaptic compound action potential (MSR AMPL) and on the integral of EPSP (EPSP INT.). Data are presented as mean ± S.E.M. from four experiments. Note that efficacy of silperisone was not dependent on the stimulus intensity, i.e., increasing the stimulus strength did not counteract the inhibition produced by silperisone.

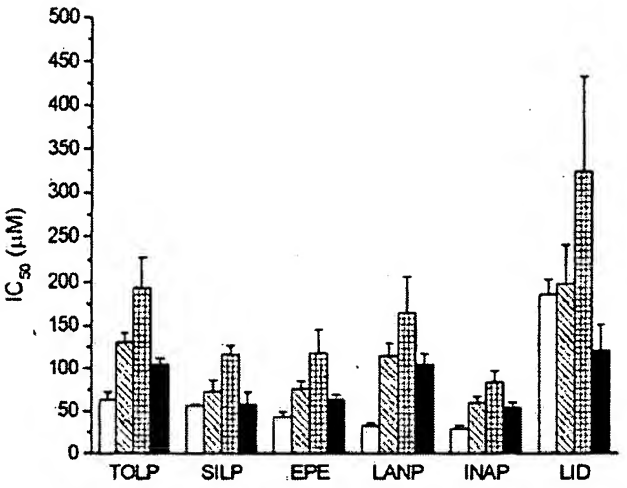

Fig. 2. Effects of different tolperisone-type centrally acting muscle relaxant drugs (tolperisone, TOLP; silperisone, SILP; eperisone, EPE; Lanperisone, LANP; and inaperisone, INAP) and lidocaine (LID) on DR-VRP components in vitro. IC_{50} values of the drugs studied are based on inhibition of different reflex components: peak-to-peak amplitude of monosynaptic compound action potential (open columns), integram of EPSP (hatched columns), amplitude of EPSP (checked columns), and integral of late part of EPSP (80–180-ms poststimulus time; solid columns). All columns represent mean ± S.E.M. from four experiments.

sensitivity order of components: MSR > DSR > PSR. Its action was longer lasting than that of tolperisone, eperisone, or lidocaine (about 40, 90, 85, and 70% recovery of MSR 1 h after i.v. injection, respectively; not illustrated). The profiles of tolperisone and eperisone (i.e., the relative sizes of effects on the three different ventral root reflex components) were practically identical. However, peak effects of tolperisone were a bit greater for all of the three components. The profile of silperisone was also similar to that of the above-mentioned two compounds. However, its peak effect on MSR was slightly weaker. The profile of lidocaine was substantially different, because it produced relatively weaker depressant effect on MSR compared with that on PSR (Fig. 4B).

Motoneuron Excitability Test in Vivo. The effects of silperisone, tolperisone, and lidocaine on excitability of motoneurons and primary afferents were studied in three to five animals. Results are shown in Fig. 5. Characteristic potentials recorded from the dorsal and ventral roots following intraspinal focal electrical stimulation of the area of the motoneuron pool are shown in Fig. 5A.

Silperisone (10 mg/kg i.v.) depressed MN (direct excitability) by 15%, whereas MS (the synaptic response) decreased by 68% on average (Fig. 5). It exerted no effect on excitability of PAFs. The onset of the effect of silperisone was relatively slow, and the inhibition reached its maximum in 15 min. Tolperisone (10 mg/kg i.v.) reached a maximum effect of 24, 80, and 8% inhibition of MN, MS, and PAF, respectively, in 6 to 8 min, whereas lidocaine (10 mg/kg i.v.) produced more marked depression of MN and PAF (30 and 14%, respectively; Fig. 5D) than did tolperisone or silperisone. On the other hand, its depressant effect on MS (67%) was less pronounced compared with tolperisone, and it was similar to that of silperisone. Although these differences may seem small, they were highly reproducible when the different compounds were administered successively in the same experiment.

Study of Afferent Nerve Conduction in Vivo. Possible contribution of a local anesthetic-type inhibition of afferent nerve conduction to the reflex inhibition following systemic

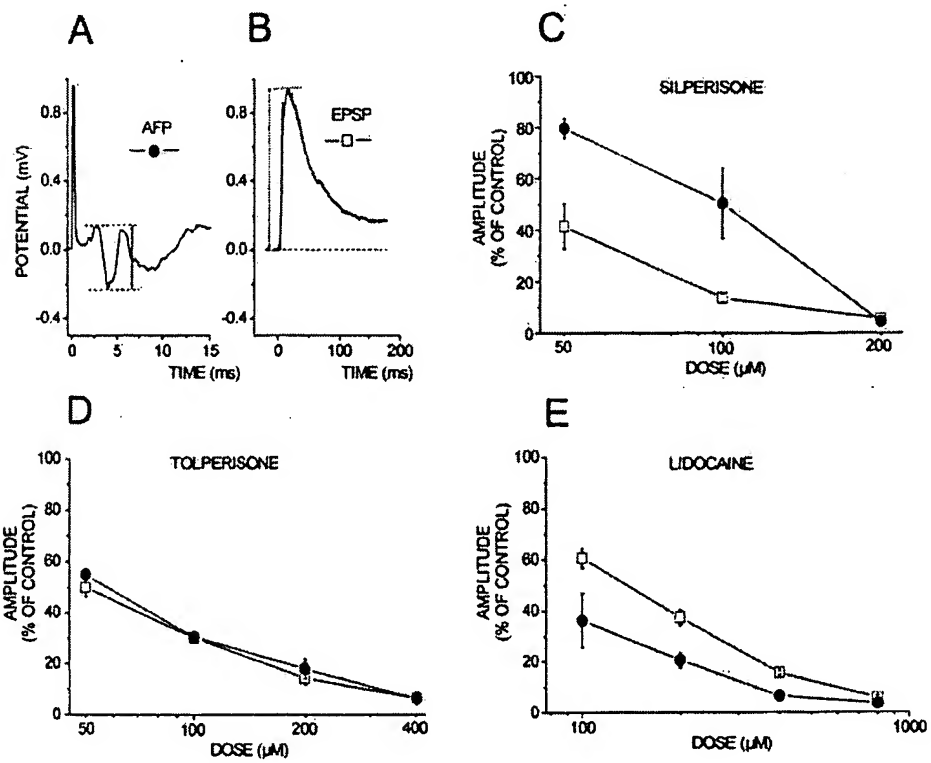

Fig. 3. Effects of drugs on synaptic responses (EPSP) and on AFP. These experiments were performed at bath temperature of 32 ± 1°C to eliminate the monosynaptic reflex and thus to record a pure EPSP. The L5 dorsal root was stimulated (0.2 mA; 0.1 ms) A, afferent fiber potential recorded from the L4 dorsal root. It shows the action potentials of L5 dorsal root arriving at the spinal cord surface. B, population EPSP recorded from the L5 ventral root. C to E, dose-response curves of silperisone, tolperisone, and lidocaine, respectively. Data points represent the peak-to-peak amplitude of AFP (●) and baseline-to-peak amplitude of EPSP (□). Data are presented as mean ± S.E.M. from three experiments, each.

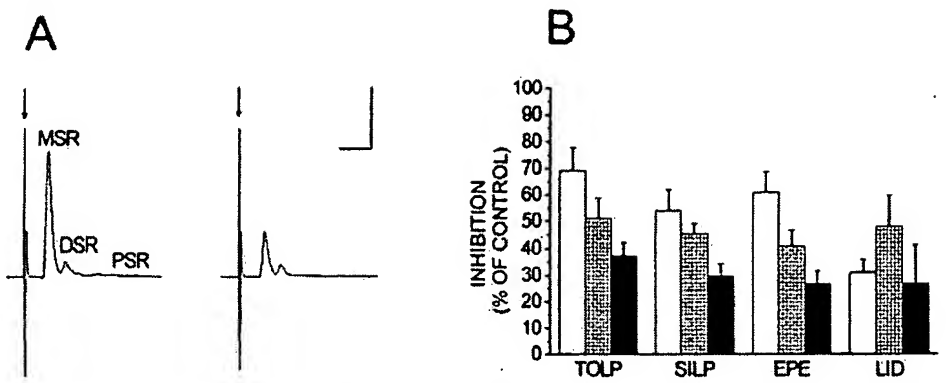

Fig. 4. Effect of drugs (tolperisone, TOLP; silperisone, SILP; eperisone, EPE; and lidocaine, LID) on ventral root reflex evoked by dorsal root stimulation recorded in vivo in spinal rats. A, representative averaged (n = 10) potential before (left) and 10 min after (right) administration of silperisone (10 mg/kg i.v.). The reflex response consists of three components: the first, robust peak, the MSR; the smaller and somewhat more variable DSR; and the longer lasting PSR, which is hardly discernible from the baseline at this amplification. The arrows indicate the stimulation artifact. Scale bars, 2 mV, 2 ms. B, maximal inhibitory effects (within 30 min after drug administration) of drugs (10 mg/kg i.v.) on different reflex components: MSR (open columns), DSR (hatched columns), and PSR (solid columns), as percentage of the control responses. Data are presented as mean ± S.E.M. from five experiments.

administration of silperisone, tolperisone, and lidocaine (10–10 mg/kg i.v.) was investigated. AFPs recorded from the L4 dorsal root following stimulation of the sciatic nerve reflected evoked action potentials of A-fibers (no synchronized C-fiber-mediated components could be recorded under our experimental conditions). Silperisone and tolperisone left AFP practically unchanged (2.7 ± 0.5 and 5.0 ± 1.2% inhibitions of the peak, respectively; n = 6). Lidocaine exerted slight but consistent inhibitory effect on AFP amplitude (10.7 ± 1.7%).

Effects on Voltage-Gated Sodium and Calcium Currents. Sodium currents recorded from medium-sized (25–35 μm) dorsal root ganglion cells had an average maximal peak current amplitude of 3.9 ± 0.4 nA (43 cells). Because in pilot experiments we found that the sodium channel inhibitory effect of tolperisone-like compounds is highly membrane potential-dependent, the concentration-response relationships were investigated at a membrane potential, where approximately half of the channels were in an inactivated state ($V_{H1/2}$), i.e., at −80 mV. Thus, to decrease the variability of drug sensitivities among DRG neurons, cells with $V_{H1/2}$ out of the range of −70 and −90 mV were not used for drug testing.

Tolperisone, silperisone, eperisone, and lidocaine inhibited peak sodium currents evoked by step depolarizations to 0 mV from a holding potential of −80 mV in a concentration-dependent manner (Fig. 6A). The inhibitory effect developed rapidly and was reversible. The IC_{50} values are listed in Table 1. Silperisone had a slightly higher potency to block sodium currents than the other three drugs.

The membrane potential dependence of the available current (sodium channel availability) was assessed by applying a voltage protocol in which membrane potential was held for 10 s at different voltages, increasing from −130 to −10 mV in 10-mV steps preceding the test pulses to 0 mV. In Fig. 6B, the effect of tolperisone (200 μM) on the steady-state inactivation curve is shown. The peak amplitude of the resultant current was normalized to the value of the maximum available current (recorded at −130 mV) and plotted against the membrane potential. Tolperisone (200 and 400 μM) as well as silperisone (80 and 160 μM) and lidocaine (200 and 400 μM) caused concentration-dependent shifts of the control inacti-

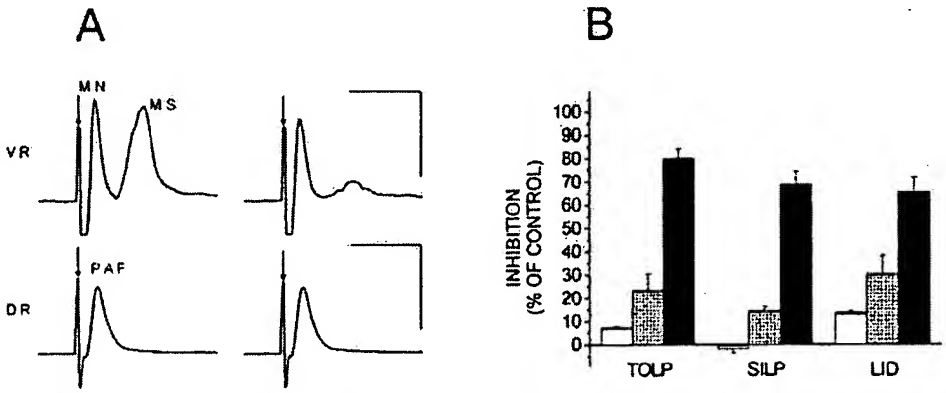

Fig. 5. Effect of drugs (tolperisone, TOLP; silperisone, SILP; and lidocaine, LID) on direct electrical excitability of MN, their MS excitation, and excitability of PAF. A, averaged potentials were recorded from L5 ventral root (VR) and from L5 dorsal root (DR), before (left) and 6 min after (right) administration of silperisone (10 mg/kg i.v.). The arrows indicate the stimulation artifact. Silperisone had negligible effect on PAF and little effect on MN, but it strongly inhibited MS. Scale bars, 1 mV, 2 ms (VR); 5 mV, 2 ms (DR). B, comparison of the effects of tolperisone (n = 3), silperisone (n = 5), and lidocaine (n = 3), each at 10 mg/kg i.v., on PAF (open columns), MN (hatched columns), and MS (solid columns). Data are presented as mean ± S.E.M.

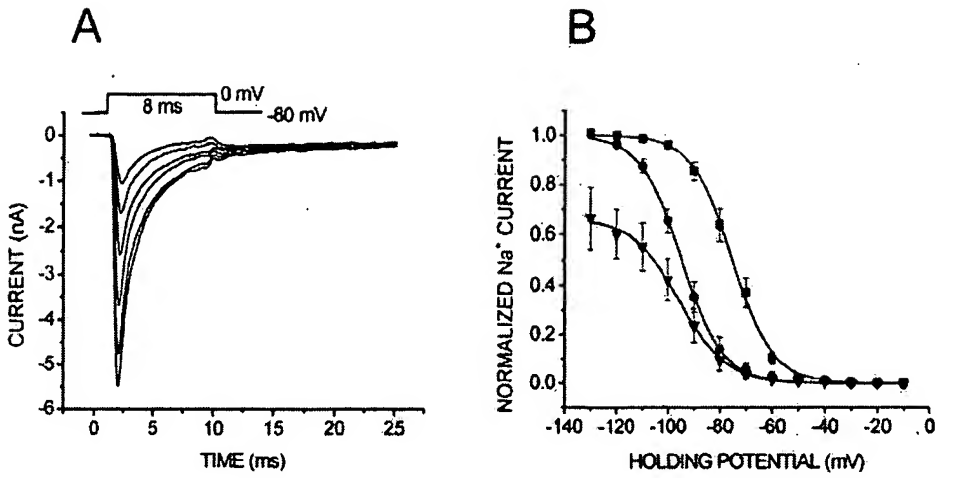

Fig. 6. Effect of tolperisone on voltage-gated sodium channels in DRG cells. A, current traces from bottom to top were recorded in the presence of 0, 40, 80, 160, 320, and 640 μM tolperisone in a representative experiment. The applied voltage command protocol is shown above the traces. B, effect of tolperisone on the steady-state inactivation characteristics. Tolperisone (200 μM; ▼) shifted the control (■) inactivation curve and decreased the maximum current at high negative potentials. The normalized curve (●) indicates a pronounced parallel leftward shift in the current availability curve of the control curve. Data are presented as mean ± S.E.M. from seven cells.

TABLE 1

IC_{50} values (micromolar; mean ± S.E.M.) of various tolperisone-type muscle relaxant drugs and lidocaine in in vitro assays
The number of observations is shown in parentheses.

Compound	Current[a]	[^{3}H]BTX Binding	VER	MSR	EPSP Integral
Tolperisone	198 ± 21 (6)	40.9 ± 2.5 (7)	58.3 ± 2.4 (3)	62.7 ± 9.4 (7)	130.7 ± 10.4 (7)
Silperisone	111 ± 18 (10)	7.2 ± 0.8 (4)	14.2 ± 0.6 (3)	55.9 ± 1.5 (4)	72.5 ± 13.8 (4)
Eperisone	250 ± 93 (5)	21.7 ± 2.5 (3)	37.1 ± 1.8 (3)	43.3 ± 5.8 (5)	76.0 ± 9.4 (5)
Inaperisone	N.D.	34.6 ± 3.0 (3)	41.8 ± 3.5 (4)	29.6 ± 3.8 (7)	59.7 ± 7.4 (7)
Lanperisone	N.D.	13.7 (1)	31.2 ± 2.9 (4)	33.0 ± 3.1 (5)	114.7 ± 15.1 (5)
Lidocaine	297 ± 86 (6)	132 ± 9 (4)	60.6 ± 6.2 (4)	186.2 ± 17.6 (6)	198.5 ± 42.8 (6)

N.D., not determined; VER, veratridine-evoked depolarization determined by microfluorimetry.
[a] Sodium currents were measured by whole-cell patch clamp.

vation curves ($V_{H1/2}$ of −75.0 ± 1.5 mV) toward the more hyperpolarized membrane potentials, but they also decreased the maximum available current (Table 2). On the contrary, none of the drugs affected the voltage dependence of activation (current-voltage curve) of sodium currents (not illustrated).

In accordance with the data of Wu and Pan (2004), we found that L-type and P/Q-type calcium channels do not contribute considerably to the high-threshold voltage-activated calcium current in DRG cells, because neither nitrendipine (10 μM; n = 4) nor ω-agatoxin-IVA (0.1 μM; n = 9) blocked the current significantly (5 ± 1 and 4 ± 1% inhibition of calcium current amplitude, respectively), whereas ω-conotoxin-GVIA, a selective N-type calcium channel blocker (3 μM), caused a 28 ± 3% inhibition (n = 4). Silperisone (320 μM) inhibited both the ω-conotoxin-sensitive current component and also most of the drug-resistant (R-type) current (Fig. 7A).

Silperisone concentration dependently inhibited voltage-sensitive calcium channels with an IC_{50} of 218 ± 22 μM (determined in eight cells). In another set of experiments, the patch pipette-filling solution lacked creatine phosphate, creatine phosphokinase, and GTP, agents that facilitate the phosphorylation of intracellular proteins. In these experiments, the blocking effect was more prominent (IC_{50} = 65 ± 9 μM; n = 8). Tolperisone had an IC_{50} of 1062 μM in the presence of creatine phosphate, creatine phosphokinase, and GTP; thus, it was ~5-fold less potent than silperisone. No IC_{50} values were determined with eperisone and lidocaine; thus, the calcium channel-inhibiting efficacies of drugs were compared at 320 μM (Fig. 7B). At their IC_{50} concentrations for sodium channel blockade, tolperisone, silperisone, eperisone, and lidocaine caused 22, 34, 36, and 7% diminution of calcium currents, respectively.

Effects on [^{3}H]BTX Binding and Veratridine-Induced Depolarization. All of the studied compounds inhibited [^{3}H]BTX binding in cortical synaptosomal preparation dose dependently. IC_{50} values are listed in Table 1. At similar concentrations, the same compounds also reduced membrane depolarization evoked by the sodium channel activator/inactivation inhibitor veratridine (Table 1). Similar to the sodium current measurements, lidocaine proved to be the least effective also in these assays. The results of the [^{3}H]BTX binding test are in good correlation with those of the veratridine depolarization assay, and the rank orders, with one exception, are in agreement with the orders found in the whole-cell current experiments. However, the potencies of the compounds in the former two assays are different from what was determined in the patch-clamp experiments. The discrepancy may be related to the fact that the former assays apply veratridine and BTX, respectively, which may alter the sensitivity of sodium channels to this type of blocking agents. Furthermore, different parameters (peak current or steady-state responses) were measured in the different assays.

Discussion

Tolperisone and its analogs as well as lidocaine suppressed the spinal segmental reflex activity both in vitro and in vivo. Various presynaptic and postsynaptic events may be considered as possible site(s) of this inhibitory action. Using an intraspinal stimulation protocol, we found that tolperisone and silperisone decreased motoneuronal (postsynaptic) excitability but to a lesser extent than the local anesthetic lido-

TABLE 2

Effects of tolperisone, silperisone, and lidocaine (micromolar) on steady-state inactivation of voltage-gated sodium channels of DRG cells

$\Delta V_{H1/2}$ is the shift of the half-maximal voltage for inactivation caused by the given drug, and I_H is the percentage inhibition of current peak amplitude at a holding potential of −130 mV. The number of observations is shown in parentheses.

Compound	$\Delta V_{H1/2}$	I_H
	mV	%
200 Tolperisone	−19 ± 3 (7)	33 ± 12 (4)
400 Tolperisone	−22 ± 3 (6)	41 ± 9 (6)
80 Silperisone	−6 ± 1 (7)	13 ± 2 (6)
160 Silperisone	−8 ± 1 (7)	32 ± 7 (6)
200 Lidocaine	−13 ± 2 (7)	14 ± 4 (6)
400 Lidocaine	−17 ± 2 (9)	27 ± 7 (7)

caine. The compound action potential recorded from the dorsal root represents excitability of primary afferents. From the ventral root, two successive potentials could be recorded: the first spike was the consequence of direct electrical stimulation (nonsynaptic excitation of the motoneurons; MN), whereas the second spike was due to MS activation of motoneurons (Ono et al., 1984; Farkas and Ono, 1995).

Lidocaine was also the most effective among the three compounds in decreasing the excitability of primary afferent terminals and the conduction of afferent volley (AFP). These presynaptic events have a great impact on the quantity of transmitter substances (primarily glutamate) released from the terminals. A decreased transmitter release results in a depression of EPSP. To analyze precisely the relationship between depression of the afferent nerve conduction and synaptic transmission, dose-response studies were performed with simultaneous recording of AFP and DR-VRP. Although tolperisone, silperisone, and lidocaine all attenuated both AFP and EPSP, silperisone preferentially depressed EPSP, whereas lidocaine possessed a more pronounced depressant action on AFP. The profile of tolperisone was between that of the two other drugs, namely, it equally inhibited AFP and EPSP.

In summary, lidocaine had significantly greater depressant effects on the direct electrical excitability of motoneurons and primary afferents than silperisone, whereas their efficacies to inhibit the synaptic transmission were similar. The profile of tolperisone was in between the two other drugs.

Among the compounds studied, silperisone had the slowest rate of onset of effect (not shown). Since an apparently complete steady state usually could not be achieved with silperisone even within 90 min, this could cause slight underestimation of its potency. As shown by Fig. 2, inaperisone was the most potent in attenuating both MSR and the early part (peak) of EPSP. However, it was equally potent to eperisone and silperisone in attenuating the "tail" and consequently the whole area under the curve of EPSP. Tolperisone and lanperisone were somewhat less potent than the above-mentioned drugs, but their patterns of actions were similar. Lidocaine was the least potent, especially in depressing the early part of EPSP. However, it was relatively potent in attenuating the tail (Fig. 2), which is in agreement with the results of Nagy and Woolf (1996) obtained in a similar model.

Thus, in spite of the fact that lidocaine had marked effects on AFP and excitability of presynaptic terminals, it was less efficient in depressing synaptic transmission compared with tolperisone and especially with silperisone. This suggests that the latter drugs may have additional inhibitory actions on EPSP generation.

In our in vitro model, EPSP could be abolished by coadministration of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid antagonist GYKI 52466 and the NMDA antagonist 2-amino-5-phosphonovalerate (Kocsis et al., 2003). However, according to our former (unpublished) studies, neither tolperisone nor silperisone had any affect on α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid or NMDA receptors, suggesting that the depressant effect on EPSP reflects a presynaptic action on the release of the excitatory transmitter rather than an action on postsynaptic glutamate receptors. Neither did the two drugs affect GABA-A receptors in binding experiments.

Voltage-gated sodium channels are regarded as one of the most important sites of action of lidocaine (Hille, 1977; Clare et al., 2000). The effect of lidocaine on sodium channels can explain both the depression of excitability and synaptic transmission observed at the spinal segmental level. Our present results clearly indicate that tolperisone and its analogs share this feature of lidocaine, which is in agreement with the findings of Ono et al. (1984), Hinck and Koppenhöfer (2001), Düring and Koppenhöfer (2001), and Quasthoff et al. (2003). The lack of clear correlation between the blocking potencies of these drugs in the tests for sodium channels and those for depression of spinal reflex transmission in vitro (Table 1) may be related to differences between the compounds regarding the diffusibility inside the spinal tissue and/or to differences in activities on other channels.

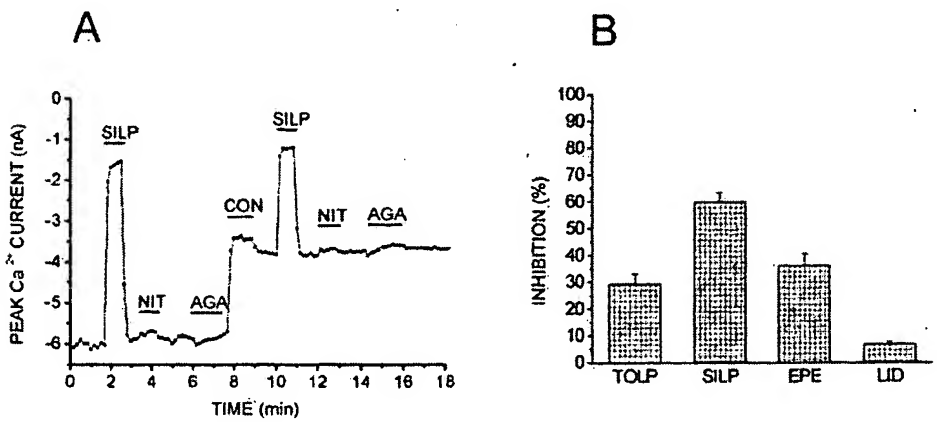

Fig. 7. Effect of drugs on voltage-gated calcium channels in DRG cells. A, an experiment illustrating the effect of silperisone (SILP; 320 μM) on high voltage-activated calcium currents compared with that of specific blockers of various subtypes of calcium channels: nitrendipine (NIT; 10 μM; L-type-selective), ω-conotoxin-GVIA (CON; 3 μM; N-type-selective), and ω-agatoxin-IVA (AGA; 0.1 μM; P-type-selective). Peaks of current were plotted against time. B, inhibitory effect of different tolperisone type centrally acting muscle relaxant drugs (tolperisone, TOLP; silperisone, SILP; and eperisone, EPE) and lidocaine (LID) at 320 μM on calcium current peak amplitude. Columns represent mean ± S.E.M. in four to seven experiments.